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Production of a Low-cost Biosurfactant for Application in the Remediation of Sea water Contaminated with Petroleum Derivates

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Biosurfactants are natural surfactants produced by bacteria, yeasts or fungi from different substrates, including sugars, oils, alkanes. Biosurfactants are expected to reach more than USD 2 billion by 2020, with industrial applications in microbial enhanced oil recovery (MEOR), removal of contamination by heavy metals, bioremediation, food, cosmetics, pharmaceuticals, biomedicine and nanotechnology. The considerable interest in these biobased products is related to their properties, as biodegradability, production from renewable substrates, low toxicity, biocompatibility, digestibility, diversity for chemical structure and properties, effectiveness even at extreme conditions of temperature, pH and salinity. Despite the advantages, fermentation must be cost-competitive with chemical synthesis, and many of the potential applications that have been considered for biosurfactants depend on whether they can be produced economically. Fermentation medium can represent approximately 30 % of the cost for a microbial fermentation. Nevertheless, much effort in process optimization at the engineering and biological levels has been done, and, for some applications, biosurfactants can be produced from several inexpensive waste substrates, thereby decreasing their production cost. Thus, the production of a biosurfactant by *Candida guilliermondii* UCP0992 was studied in a low-cost medium formulated with 2.5 % waste frying oil, 2.5 % corn steep liquor and 4.0 % molasses during 144 h at 28 ºC under 200 rpm. The surface tension of the medium was reduced to below 28 mN/m. The tensoative properties of biosurfactant were investigated and its CMC (Critical Micelle Concentration) determined as 0.42 %. It had a good surface tension reduction capacity and emulsifying activity against motor oil (up to 90 %) and vegetable oils (30-55 %). It showed stability during exposure to high temperatures (up to 120 °C for 15 min), high salinity (12 % NaCl) and a wide range of pH (2-12). The crude biosurfactant did not show toxicity against the microcrustacean Artemia salina. The biosurfactant was also tested for toxicity against bacteria and filamentous fungi from seawater during 30 days. The addition of the biosurfactant to seawater stimulated the degradation of motor oil via the activity of the indigenous microorganisms. The cell-free broth (crude biosurfactant) was also effective in oil displacement (100 %) in seawater. The biosurfactant from *C. guilliermondii* was also effective in recovery of up to 50 % motor oil from the walls of beakers at twice its CMC. These results indicate the potential value of the biosurfactant for application in the oil industry, especially in enhanced oil recovery, tank cleaning and in bioremediation of spills at seas and soils.

1. Introduction

Biosurfactants are natural surfactants produced by bacteria, yeasts or fungi from different substrates, including sugars, oils, alkanes. Microbial surfactants fall into two categories: (i) low molecular weightbiosurfactants, which include glycolipids, lipopeptides, peptides, fatty acids and neutral lipids, and (ii) high molecular weightbiosurfactants composed of polysaccharides, proteins, lipopolysac-charides, lipoproteins or complex mixtures of these biopolymers (Silva et al., 2014).
Biosurfactants are expected to reach more than USD 2 billion by 2020, with industrial applications in microbial enhanced oil recovery (MEOR), removal of contamination by heavy metals, bioremediation, food, cosmetics, pharmaceuticals, biomedicine and nanotechnology (Sarubbo et al., 2015a). The considerable interest in these

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biobased products is related to their properties, as biodegradability, production from renewable substrates, low toxicity, biocompatibility, digestibility, diversity for chemical structure and properties, effectiveness even at extreme conditions of temperature, pH and salinity (Sarubbo et al., 2015b).
Despite the advantages, fermentation must be cost-competitive with chemical synthesis, and many of the potential applications that have been considered for biosurfactants depend on whether they can be produced economically. Nevertheless, much effort in process optimization at the engineering and biological levels has been done, and, for some applications, biosurfactants can be produced from several inexpensive waste substrates, thereby decreasing their production cost (Silva et al., 2014). A great variety of alternative raw materials is currently available as nutrients for industrial fermentations, namely various agricultural and industrial byproducts and waste materials.
Thus, environmental and economic issues have motivated the completion of this study that presents biosurfactant production by the yeast *Candida guilliermondii* (Brasileiro et al., 2015) using a low-cost medium supplemented with waste frying soy oil, molasses and corn steep liquor as substrates. This study also describes the biosurfactant characterization, surface active properties, emulsifying capacity and toxicity. The application of the biosurfactant in the environment was also investigated.

2. Materials And Methods

2.1 Materials and microorganism

The waste frying oil used was obtained in a restaurant located in the city of Recife (Brazil). Corn steep liquor was obtained from Corn Products do Brasil in the city of Cabo de Santo Agostinho (Brazil) and corn steep liquor from a Sugar cane Factory in Belo Jardim city, (Brazil).
Candida guilliermondii UCP0992 was obtained from the culture collection of the *Universidade Católica de Pernambuco* (Brazil). The microorganism was maintained at 5 °C on yeast mould agar slants containing (w/v) yeast extract (0.3 %), malt extract (0.3 %), tryptone (0.5 %), D-glucose (1.0 %) and agar (5.0 %). Transfers were made to fresh agar slants each month to maintain viability.

2.2 Growth conditions

The inoculum of *Candida guilliermondii* was prepared by transferring cells grown on a slant to 50 ml of yeast mould broth. The seed culture was incubated for 24 h at 28 °C and agitated at 150 rpm. The yeast was cultivated in a submerged culture with agitation in an orbital shaker. The production medium contained 2.5 % wast frying oil, 2.5 % molasses and 4.0 % corn steep liquor. The medium was sterilised by autoclaving at 121 °C for 20 min (all components were sterilised together). The final pH of the medium was 5.3. The inoculum (1 % v/v) was introduced in the amount of 10^4 cells/ml to a cool medium yeast.

2.3 Biosurfactant production

Fermentation was carried out in 500 mL Erlenmeyer flasks with a 100 ml working volume. For inoculation, the flasks were allowed to cool down to room temperature (27 °C) before transferring 1 % (v/v) primary inocula of the cell suspension into the production media. The cultures were incubated in a rotary shaker for 144 h at 200 rpm. There was no adjustment of pH during cultivation.

2.4 Biosurfactant isolation

The biosurfactant was extracted from culture media after cell removal by centrifugation at 4500 g for 20 min. The supernatant pH was adjusted to 2.0 with 6.0 M HCl, and precipitaded with methanol. After 48 h, the mixture was centrifuged again and maintained at 37 ºC until constant weight.

2.5 Surface tension and CMC determination

The surface tension of the culture supernatants obtained by centrifuging the cultures at 5000 g for 20 min was measured using a Sigma 700 digital surface tensiometer (KSV Instruments LTD - Finland) working on the principle of the *Du Nuoy* ring method.
The critical micelle concentration (CMC) was determined by measuring the surface tensions of dilutions of isolated biosurfactant in distilled water up to a constant value of surface tension. Stabilization was allowed to occur until standard deviation of 10 successive measurements was less than 0.4 mN/m. Each result was the average of 10 determinations after stabilization. The value of CMC was obtained from the plot of surface tension against surfactant concentration. The CMC value was determined to be g/L of biosurfactant.

2.6 Emulsifying activity with different hydrophobic compounds

Emulsification index (EI) was measured using the method described by Cooper and Goldenberg (1987), whereby 2 ml of a liquid hydrophobic compound (motor oil, diesel, and vegetables oils) was added to 2 ml of the culture broth free of cells in a graduated screwcap test tube, and vortexed at high speed for 2 min. The

emulsion stability was determined after 24 h and the emulsification index was calculated by dividing the measured height of the emulsion layer by the mixture's total height and multiplying by 100.

2.7 Effect of environmental factors on biosurfactant activity

The effect of addition of different concentrations of NaCl on the activity of the biosurfactant was investigated in the cell-free broth. A specific concentration of NaCl (2-12 %, w/v) was added, and surface tension and emulsification activity were determined as previously stated. The cell-free broth was also maintained at a constant temperature (0, 10, 28, 50, 100 and 120 °C) for 60 min and used for surface tension and emulsification measurements. The effect of pH on surface tension and emulsification was evaluated after adjustment of the broth pH to 2, 4, 6, 8, 10 and 12 with 6.0 M NaOH or HCl.

2.8 Biochemical composition of the biosurfactant

Protein concentration in the isolated biosurfactant was determined by the *Labtest Diagnóstica S.A. Brasil* kit using Bovine serum albumin as a standard. Carbohydrates were determined according to Dubois et al. (1956). The lipid composition of the isolated biosurfactant was determined according to Manocha et al. (1980).

2.9 *Artemia* assay

The toxicity assay was performed using brine shrimp (*Artemia salina*) as the toxicity indicator. Brine shrimp eggs were obtained from a local store. Larvae were used within one day of hatching. Following dilutions of a biosurfactant solution at 1/2CMC and at the CMC with saline water (33 g/L) to give concentrations of 25 %, 50 % and 75 %, the assays were conducted in 10 ml penicillin tubes containing 10 brine shrimp larvae in 5 ml of saline water per tube. The brine shrimp larvae in each tube were tested using 5 ml of each concentration of the biosurfactant solution. The samples were observed for 24 h for the calculation of the mortality rate (Meyer et al., 1982). The toxicity threshold concentration, expressed as biosurfactant concentration per 100 ml of saline water, was defined as the lowest concentration that killed all brine shrimp within 24 h. Each test was run in triplicate and saline water was used as the control.

2.10 Application of the biosurfactant in hydrophobic contaminant spreading

The oil displacement test was carried out slowly by dropping of 15 µl of motor oil onto the surface of 40 ml of distilled water layer contained in a Petri dish (15 cm in diameter) that spread all over the water surface area. This was followed with the addition of 10 µl of the cell-free broth or aqueous solutions containing the isolated surfactant at 1/2CMC and at the CMC onto the surface of the oil layer. The average value of the diameters of the clear zones of triplicate experiments was measured and recorded then calculated as percentage of the Petri dish diameter (Saeki et al., 2009).

2.11 Application of the biosurfactant in hydrophobic contaminant cleaning test

As a means to check the cleaning ability of the biosurfactant, the inner walls of a set of beakers were coated with motor oil. To remove the adhered oil, 50.0 ml of the cell-free broth or wash solutions containing the isolated biosurfactant at 1/2CMC and at the CMC was added to each beaker, vortexed for 1 min, and allowed to stand for 6 h (Pruthi and Cameotra, 2000).

2.12 Bioremediation test

Bioremediation tests were performed according to the method mentioned in the Standard Methods for the Examination of Water and Wasterwater (APHA, 2005). In brief, 250-mL Erlenmeyer flasks were filled with 100 mL fresh seawater obtained from the Suape Petrochemical Complex, Pernambuco State, Brazil, 1 % of motor oil, and biosurfactant solutions at concentrations of 1/2CMC and at the CMC. Control flasks contained seawater and motor oil. The flasks were incubated at 28 °C on an orbital shaker rotating at 150 rpm. Triplicate shake flasks were sacrificed on days 1, 5, 7, 21 and 30 of incubation and then analyzed for the number of microorganisms by using the most probable number (MPN).

3. Results and Discussion

3.1 Surface tension, CMC and biosurfactant yield

The yield of the biosurfactant produced by *C. guilliermondii* was 2.15 g/L and the surface tension of the medium was reduced from 50 mN/m to 28 mN/m. Rufino et al. (2007) showed similar results for the biosurfactant produced by *C. lipolytica* using refinery residue as substrate.

The biosurfactant produced exhibited excellent ability of reducing the surface tension, since the surface tension of the water was reduced from 71 mN/m to 28.0 mN/m at Critical Micelle Concentration (CMC) of 0.42 % (Figure 1).

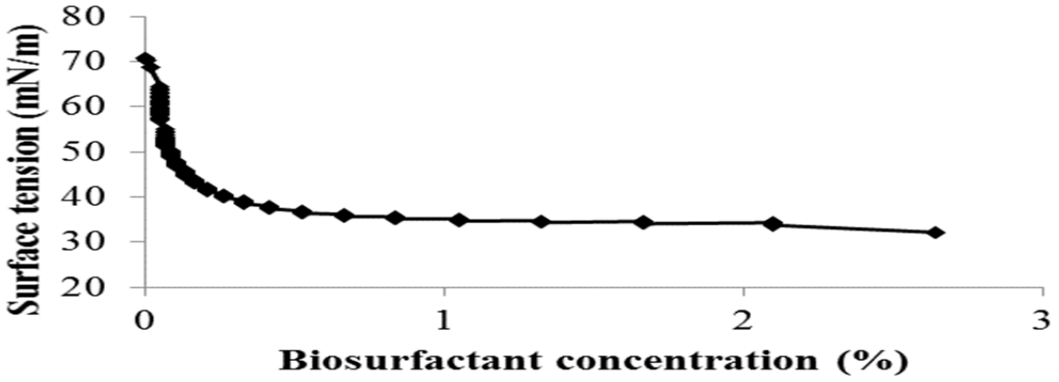

Figure 01: Surface tension versus concentration of the isolated biosurfactant from C. guilliermondii cultivated in medium supplemented with 2.5 % waste frying oil, 2.5 % corn steep liquor and 4.0 % molasses

From this point, the concentration of the biosurfactant solution did not cause further reductions in the surface tension of water, indicating that the CMC had been reached at this concentration.
The biosurfactant from *C. guilliermondii* showed ability to reduce the tension better than the biosurfactants from *C. lipolytica* (32 mN/m) (Rufino et al. 2007) and *C. glabrata* (31 mN/m) (Sarubbo et al., 2006).

3.2 Biochemical composition of the biosurfactant

Preliminary analysis of the biosurfactant identified the presence of 12.4 % lipids, 52.3 % protein and 10.0 % carbohydrate, while the Liposan produced by *C. lipolytica* using hexadecane as the substrate consists of 83% carbohydrates and 17 % protein (Cirigliano and Carman, 1985). Another biopolymer produced by *C. lipolytica* using glucose as substrate showed 47 % protein, 45 % carbohydrate and lipid 5 % (Sarubbo et al., 2001) and the bioemulsifier obtained using brazilian babassu oil as substrate showed 60 % carbohydrates, 23 % protein and 11 % lipids, demonstrating that the same micro-organism can produce different biosurfactants depending on the used substrate (Sarubbo et al., 1999).

3.3 Effect of environmental factors on biosurfactant activity

Despite the diversity of chemical composition and properties, some features are common to most biosurfactants. Many of these features represent advantages over conventional surfactants: tolerance to temperature, pH and ionic strength (Silva et al., 2014). Table 1 shows the biosurfactant resistance against different pH values (2, 4, 6, 8, 10 and 12), demonstrating that its stability was not affected even when subjected to extreme pH values. As for stability, biosurfactant revealed surface tension values, which ranged 28 to 32 mN/m, regardless the NaCl concentration to which it was submitted. The biosurfactant stability testing against different temperatures (0, 10, 28, 50, 100 and 120 °C) was also conducted, demonstrating that its stability was altered when subjected to temperatures of 0 and 10 °C, although to temperatures above 28 °C its surface tension showed no significant changes, indicating the possibility of using the biosurfactant for control of environment pollution caused by oily compounds in at different temperatures.
In addition to surface tension, the stabilization of oil and water emulsions are commonly used as surface activity indicator. The emulsification activity of the biosurfactant produced was determined for various immiscible substrates in water under extreme environmental conditions (Table 1). High emulsion values were obtained for the motor oil (above 50 %) when tested at concentrations up to 4 % NaCl. Stable emulsions were formed for motor oil independent of the pH range tested.
Vegetable oils of corn and soybean showed good emulsification activity (35 to 45 %), regardless the quantity of NaCl and pH values which thy have been subjected, while sunflower oil showed emulsion formation between 30-40 % under the conditions tested. Diesel was not effectively emulsified by the biosurfactant. These results indicate that the biosurfactant produced has specificity for emulsifying the lubricating motor oil and the vegetable oils tested. Similar results were obtained for *Rhodococcus* grown on hydrocarbons (Abu-ruwaida et al., 1991). The results confirm that the emulsifying activity depends on the affinity of the biosurfactant by the tested hydrocarbon.
With respect to temperature, motor oil and diesel oil were less emulsified by the biosurfactant with the increase in temperature above 100 °C, while the vegetable oils produced more stable emulsions, with an emulsifying variation of 10 % starting from the mean values.

Table 1: Influence of salt concentration, temperature and pH on the surface tension reducing activity and on the emulsifying activity of the cell-free broth containing the biosurfactant from C. guilliermondii cultivated in medium supplemented with 2.5 % waste frying oil, 2.5 % molasses and 4.0 % corn steep liquor

NaCl (%)	Surface tension (mN/m)	EI (%)[a]	EI (%)[b]	EI (%)[c]	EI (%)[d]	EI (%)[e]
0	28	56	45	30	38	10
2.0	31	56	40	30	30	10
4.0	28	90	50	36	43	6
6.0	31	4	40	40	40	7
8.0	32	5	40	40	40	18
10.0	32	5	45	40	43	20
12.0	32	3	45	40	43	25
Temperature (°C)	**Surface tension (mN/m)**	**EI (%)[a]**	**EI (%)[b]**	**EI (%)[c]**	**EI (%)[d]**	**EI (%)[e]**
0	43	37	43	39	35	35
10	37	48	46	43	45	44
28	32	34	40	33	43	30
50	31	40	40	38	37	43
100	28	25	55	40	45	25
120	31	30	55	43	45	30
pH	**Surface tension (mN/m)**	**EI (%)[a]**	**EI (%)[b]**	**EI (%)[c]**	**EI (%)[d]**	**EI (%)[e]**
2	37	55	58	35	35	15
4	35	55	39	30	40	25
6	32	50	42	20	38	23
8	28	65	38	38	38	10
10	31	60	38	38	40	15
12	31	56	38	38	40	9

[a]Emulsification index of motor oil; [b]Emulsification index of corn oil; [c]Emulsification index of sunflower oil; [d]Emulsification index of soybean oil; [e]Emulsification index of diesel.

3.4 *Artemia* assay

The biosurfactant produced by *C. guilliermondii* showed no toxicity against the micro-crustacean *Artemia salina* independent of the concentrations used (1/2xCMC, CMC). In studies, toxicity tests showed that the biosurfactant caused 100 and 50 % lethality when tested at high concentrations of 700 and 525 mg/L, respectively.

3.5 Application of the biosurfactant in hydrophobic contaminant spreading

The results obtained showed values of 100 %, dispersion of the oily compound in seawater after the addition of the cell-free broth, while the isolated biosurfactant was not effective in dispersing the motor oil.

3.6 Application of the biosurfactant in hydrophobic contaminant cleaning test

After stirring for 10 minutes and 24 h of rest, it was observed removals of 40 % and 50 % of the oil from the beaker walls by the biosurfactant at concentrations of 1/2xCMC and CMC, respectively.

3.7 Bioremediation test

The results presented in Figure 02 demonstrated that, regardless of the biosurfactant concentrations used in medium containing seawater with or without oil, it showed no toxicity to the indigenous microorganisms from seawater, as they exhibited growth during the 30 days cultivation.

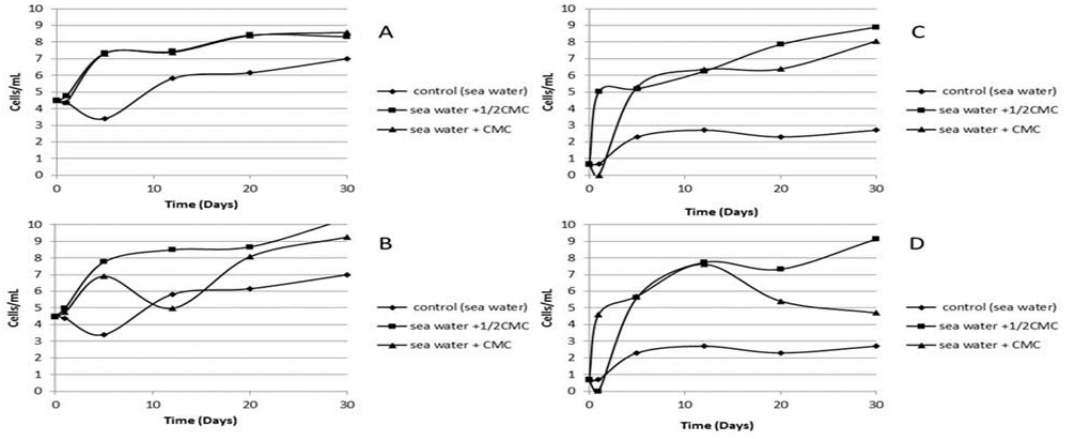

Figure 02: Influence of the biosurfactant from C. guilliermondii on the growth of: (a) indigenous bacteria in sea water without petroleum; (b) indigenous bacteria in sea water with petroleum; (c) filamentous fungi in sea water without petroleum and (d) filamentous fungi in sea water with petroleum

4. Conclusion

The biosurfactant produced by *C. guilliermondii* cultivated in low cost medium formulated with soybean frying oil, molasses and corn steep liquor showed emulsifiers and surface-active attractive features. The use of a low cost medium through showed to be promise for industrial use and the biopolymer produced can be considered a promising agent for use in the control of environmental pollution caused by oily compounds spilled in seawater. The results of the removal of oily stains experiments under different contamination conditions clearly demonstrate the viability of application of this biomolecule as an additive for remediation processes that consider the preservation and the reduction of environmental impacts on ecosystems water, essential aspects for maintaining quality of life.

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